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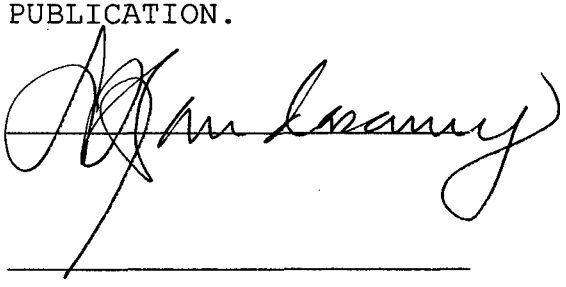

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13. ABSTRACT *(Maximum 200 Words)*

Tumor growth is absolutely dependent upon angiogenesis. Prior to the development of breast cancer, the breast tissue in many women undergoes progressive changes, which include proliferative breast disease and carcinoma in situ. We examined these pathologic changes for the level of vascularity and the presence of angiogenic growth factors. Our data show that angiogenesis begins in the earliest stages of progression. In addition, even normal breast tissue contains a complex mixture of angiogenic factors that increase in the epithelium, stroma, or infiltrating leukocytes during progression to invasive breast cancer. Using an in vitro organ culture system of normal breast tissue, we found that exogenous angiogenic factors were able to stimulate endothelial cell proliferation but not sprouting. Furthermore, under non-stimulated condition, endothelial cell proliferation was restricted to the adipose tissue and peri-lobular connective tissue. Endothelium within the fibrous stroma could almost never be induced to proliferate. However, isolated normal tissue- or tumor fibroblasts were not able to inhibit growth factor-induced angiogenesis in vitro. Our data suggest that angiogenesis is a potential chemopreventive target for the prevention of invasive breast cancer.

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Angiogenesis, In Vitro Model, Human Tissue, Growth Factors, Prognosis, Tissue Culture, Humans, Anatomical Samples, Breast Cancer, Therapy

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PI - Signature 9/25/92 Date

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INTRODUCTION

This report covers the one-year continuation of DAMD17-94-J-4271. Due to technical difficulties, the proposed SOW could not be completed within the four-year time allotment. In particular, the proposed organ culture system proved to be too insensitive to measure the changes in angiogenesis required for testing our hypotheses and another in vitro system was developed, as explained in our final report. In addition, we needed to acquire sufficient human surgical material to test the hypothesis that normal mammary epithelium differed in its angiogenic potential depending upon the patient source.

Below we describe the results from our proposed SOW, which were outlined in our letter requesting extension (dated 7/6/98) and are reiterated below. In addition, this report revisits SOW that were completed or in which new information has developed. Appropriate manuscripts are attached.

Briefly, the focus of the funded research was to examine human pre-invasive breast pathologies in archival tissues for their degree of vascularization, develop in vitro techniques and test whether any identified alterations in vascularity could be due to angiogenesis, look for the presence of factors that may regulate vascularity in these tissues, and develop a breast organ culture system in which angiogenic inhibitors may be tested as potential chemopreventive agents. Our studies showed that pre-invasive breast disease has an increased vascularity with progression. In addition, vascularity correlates with histological features determining risk of subsequently developing invasive disease. Below we show data suggesting that this increase in vascularity may be due to angiogenesis. Our work documenting the expression of angiogenic growth factors and the expression of oncogenes and tumor suppressor genes in this set of pathologies indicates that vascularity is regulated by complex mechanisms that are dependent upon the genetic pathway a given pathology follows toward progression to invasion. As described in our final report, the organ culture system we developed was not sensitive enough to measure changes in angiogenesis on a consistent basis. However, we have developed other in vitro systems and have begun to test the role stromal cells play in regulating angiogenesis in breast tissue. Finally, our studies have led us to a functional question, ie. does the increase in vascularization provide a functional limitation to the development of invasive tumors. The data provided by this grant proposal provided the foundation for subsequent funding from the National Cancer Institute to test this functional question.

BODY

Specific Aim 1: Identify and quantify angiogenesis in the earliest stages of breast carcinoma and correlate these data with clinical outcome. This project was completed and published, as indicated in our final report.

Specific Aim 2: Localize to specific cellular subsets known angiogenic factors in in situ carcinoma and atypical hyperproliferative lesions of the breast. In addition to the data presented in our final report, attached is a preprint of an article containing these data, which is in press (1).

During the past five years, several groups have been examining the role oncogenes and tumor suppressor genes play in regulating the angiogenic phenotype of tumors. Therefore, we selected a number of the oncogenes and tumor suppressor genes, known to be important in breast tumor formation, and examined the protein expression of these genes in pre-invasive breast pathologies of known vascularity. These data are summarized in the enclosed manuscript that has been submitted for publication and is currently in review (2).

Specific Aim 3: Develop an in vitro breast organ culture system and test whether the angiogenic agents identified in Specific Aim 2 induce angiogenesis in this system. As summarized in our final report, the proposed organ culture system was not sufficiently reproducible to identify known angiogenic inhibitors and therefore, it was not sufficient for our studies. However, there were experiments that suggested that fibroblast-mammary tissue co-cultures had less angiogenesis than mammary tissue organ cultures alone.

Therefore, for this one year extension we tested the following hypotheses (see letter for extension page 3):
1. Fibroblasts derived from normal breast tissue can inhibit the activity of angiogenic stimulators such as bFGF.

2. Fibroblasts derived from tumors do not inhibit angiogenesis.
3. Epithelium from either normal or tumor specimens is angiogenic when isolated from its stromal elements.

Task 1 and 2: Fibroblasts from normal breast tissue (isolated from both reduction mammoplasties and mastectomies) and were tested for their ability to induce endothelial cell tubule formation, a measure of in vitro angiogenesis, and for their ability to inhibit basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF)-induced endothelial tubule formation.

Method: Assays are performed in 48 well plates. 30,000 HUVEC (Clonetics) are plated on 175 µl of gelled type I collagen (Bectin Dickinson) in defined media (EBM, Clonetics). Two hours later, the cells are overlayed with another 175 µl of collagen. As soon as the collagen is solid, media is placed on the sandwich. If the test reagent is conditioned media, it is mixed 1:1 with the HUVEC basal media. If tissue is tested, the minced tissue or isolated epithelial organoids are placed directly on the upper layer of collagen. If cells are tested they are plated at confluency on 8 µm pore transwells 18 hours prior to placing the transwell over the collagen sandwich. Seven hours following addition of the test agent to the collagen sandwich assay, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, Sigma) is added to a final concentration of 1mg/ml. Live cells take up this vital dye and form an insoluble formazan crystal, which stains the vessels blue. Tiff files are collected from each well nine hours after the addition of media. Each condition is run in triplicate. Mean vascular areas and lengths are calculated for each well. 50 ng/ml bFGF is run as a positive control in each assay. Data are normalized to medium (0%) and bFGF (100%).

Results: Figure 1 shows the results of experiments testing cultured normal fibroblasts (A-C) derived from reduction mammoplasty (A) or mastectomy (B and C) or from fresh tumor explants (D and E). To compare among experiments the endothelial tubule length data are normalized to medium without angiogenic growth factors (0%) and medium with 50 ng/ml bFGF (100%). In no case did these cells elicit an angiogenic response greater that medium alone. Furthermore, they were all statistically less angiogenic than bFGF ($p<0.05$).

To test whether fibroblasts could inhibit growth factor induced angiogenesis, additional fibroblasts from additional reductions, mastectomies and tumors were obtained. Figure 2 shows that none of these fibroblasts were able to inhibit bFGF-induced angiogenesis (A and B= normal; C and D = tumor) in that none of these vascularities is statistically different from bFGF alone. Figure 3 shows similar data for VEGF.

Conclusion: 1. Neither fibroblasts from normal breast tissue (any source) nor from tumors are angiogenic in vitro. 2. Fibroblasts are not able to inhibit bFGF or VEGF-induced angiogenesis.

Task 3. Determine whether epithelium from either normal or tumor specimens is angiogenic when isolated from its stromal elements.

Methods: Epithelial organoids were isolated from finely minced normal mammary tissue by incubation for 18-36 hours in 150 U/ml Collagenase Type I (Gibco-BRL, Great Plains, NY), 100 U/ml Hyaluronidase (Gibco-BRL, Great Plains, NY) in CDM3 medium. CDM3 medium consists of DMEM:F12 (1:1), 2.6 ng/ml selenium, 100 ng/ml Epidermal Growth Factor, 0.1 mg/ml fibronectin (all from Collaborative Biomedical Products, Bedford, MA), 3 mg/ml insulin, 25 mg/ml transferrin, 10^{-10} M estradiol, 1.4 x10^{-6} M hydrocortisone, 10^{-8} M cAMP, 10^{-8} M triiodothyronine, 10^{-4} M ethanoloamine, 10^{-4} M phosphoethanolamine, 0.01% (w/v) BSA, 10 mg/ml ascorbic acid, 20 mg/ml fetuin (Sigma Chemical, St. Louis, MO), and1x trace element mix (BioFluids, Rockville, MD). Following digestion, epithelial organoids were separated from vascular fragments and mesenchymal cells by gravity sedimentation at room temperature. Organoids were then placed directly into the tubule formation assay. Endothelial tubule formation was assayed as described above.

Results: Figure 4 shows that the angiogenic capacity of human breast tumor pieces is equivalent to that of 50 ng/ml basic fibroblast growth factor. Shown are mean data from tumors from two patients. Skeletal muscle pieces were used as a "normal" tissue controls and are statistically different from either the tumors

or bFGF ($p<0.05$). Tumor cells and cell lines give similar results. These data confirm that indeed human tumor cells are angiogenic in our assay system.

More important are the results from isolated normal human breast epithelial organoids. Organoids are isolated ducts and lobules that are primarily epithelial with no extra-lobular stromal elements. As predicted from our examination of the vascularity in archival tissues (3), normal epithelium from women who have invasive breast cancer is more vascular than normal epithelium from women who do not have breast cancer (from reduction mammoplasties). Because breast cancer is a segmental disease, we also tested whether the vascularity of normal epithelium differs if it is derived from adjacent to the tumor (Mast(s): same quadrant) or from a region of the breast distant from the tumor (Mast (o): other quadrant). Figure 5 shows that the only epithelium that is angiogenic is the epithelium that lies adjacent to invasive epithelium. Shown are mean data derived from organoids from 10 patients in each category. Each isolate of tissue was examined histologically to exclude the possibility that our specimens included invasive tumor cells. In addition, histologic sections of the area all around the tumor were examined to preclude the testing of other ductal or lobular pathologies.

Conclusion: 1. Tumor epithelium is angiogenic in vitro. 2. Normal breast epithelium is only angiogenic when isolated from anatomical sites adjacent to invasive disease.

KEY RESEARCH ACCOMPLISHMENTS:

- Breast tumor formation is accompanied by an increase in vascularization that correlates with histological features conferring risk of subsequent invasive disease.
- Normal breast epithelium is more vascular in women who have or subsequently will have invasive disease relative to women who do not have invasive disease.
- Normal breast epithelium is uniquely angiogenic in vitro only when isolated from quadrants containing invasive tumor.
- Many angiogenic growth factors are present during tumorigenesis and most growth factors increase in their level of expression during tumorigenesis particularly at the stage of carcinoma in situ.
- Angiogenesis cannot be produced in breast organ cultures in vitro; however, the endothelium in these cultures proliferates upon the application of numerous angiogenic growth factors.
- Fibroblasts inhibit vascular density in breast organ cultures, but isolated fibroblasts from either normal breast tissue or from breast tumors cannot inhibit bFGF or VEGF-induced endothelial cell tubule formation in vitro.
- Ductal carcinoma in situ of the breast is highly vascularized in vivo. The degree of vascularization correlates directly with p53 immunoreactivity and indirectly with cyclin D1 immunoreactivity.
- Breast organ cultures of normal tissue from pre vs. post-menopausal women show that post-menopausal epithelium has a unique capacity to undergo spontaneous squamous differentiation.

REPORTABLE OUTCOMES

Heffelfinger SC, Yassin R, Miller MA, Lower E. Vascularity of proliferative breast disease and carcinoma in situ correlates with histologic features. Clinical Cancer Res, 2:1873-1878, 1996.

Heffelfinger SC, Miller MA, Gear RB, DeVoe G. Staurosporine-induced versus spontaneous squamous metaplasia in pre- and post-menopausal breast tissue. J Cell Physiol 176: 245-254, 1998.

Heffelfinger SC, Miller MA, Yassin R, Gear R. Angiogenic growth factors in pre-invasive breast disease, Clinical Cancer Res, 1999, in press.

Heffelfinger SC, Yassin R, Miller MA, Lower EE. Cyclin D1, retinoblastoma, p53, and Her2/neu protein expression in pre-invasive breast pathologies: correlation with vascularity. 1999, in review.

Taylor KL, Gear RL, Miller MA, Heffelfinger SC. Increased angiogenic capacity of normal breast epithelium isolated from women with invasive breast cancer, 1999, in preparation.

NCI, PI, Mammary Tumor Chemoprevention by Angiogenic Inhibitors, 40% effort, 7/1/99-6/30/04, $1,855,644.

CONCLUSIONS

One year report summary: This report follows our "final" report from 1998 to provide the additional data gained following a "no-cost" extension for one year. These experiments were based on data from whole mammary tissue organ cultures that suggested that fibroblasts from normal tissues might inhibit angiogenesis. Fibroblasts isolated from normal human mammary tissue and from human invasive ductal carcinomas were not able to inhibit either bFGF or VEGF-induced endothelial cell tubule formation in vitro. Furthermore, we were able to show that fibroblasts from neither source are angiogenic themselves. These data suggest that tumor-induced angiogenesis is due to products derived from the tumor epithelium with little influence from the surrounding stroma. Finally, and most importantly, we showed that angiogenesis occurs very early in the process of tumor formation and that histologically normal epithelium sharing the same quadrant as invasive tumors is also angiogenic in vitro. These data provide the basis of continued work to determine whether tumor formation is dependent upon angiogenesis.

Final report summary: Data from these studies indicate that angiogenesis is a phenotype of genetically damaged epithelium, as is seen in pre-invasive breast pathologies. Even histologically normal epithelium, when adjacent to invasive disease, is angiogenic in vitro and has an increased vascularity in vivo. The increase in vascularity that occurs during disease progression correlates with the presence of a number of angiogenic growth factors and the mutation/overexpression of genes that may regulate angiogenesis.

So what: The major question yet to be asked is whether disease progression is dependent upon angiogenesis, ie. if one inhibited angiogenesis early in the process of tumorigenesis, would one still develop invasive disease. This question is being addressed in subsequent work now funded by the NCI. If disease progression is dependent upon angiogenesis, then angiogenesis is a potential chemopreventive target for women at high risk of developing invasive disease.

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1. Heffelfinger SC, Miller MA, Yassin R, Gear R. Angiogenic growth factors in pre-invasive breast disease, Clinical Cancer Res, 1999, in press.
2. Heffelfinger SC, Yassin R, Miller MA, Lower EE. Cyclin D1, retinoblastoma, p53, and Her2/neu protein expression in pre-invasive breast pathologies: correlation with vascularity, 1999, in review.
3. Heffelfinger SC, Yassin R, Miller MA, Lower E. Vascularity of proliferative breast disease and carcinoma in situ correlates with histologic features. Clinical Cancer Res, 2:1873-1878, 1996.

APPENDICES

Appendix 1. Figures 1-5.
Appendix 2. Abbreviations
Appendix 3.
Heffelfinger SC, Miller MA, Yassin R, Gear R. Angiogenic growth factors in pre-invasive breast disease, Clinical Cancer Res, 1999, in press.

Heffelfinger SC, Yassin R, Miller MA, Lower EE. Cyclin D1, retinoblastoma, p53, and Her2/neu protein expression in pre-invasive breast pathologies: correlation with vascularity. 1999, in review.

Figure 1 shows the degree of in vitro endothelial cell tubule formation induced by cultured fibroblasts derived from normal breast tissue (A-C) or from tumors (D-E). All data were normalized to medium alone (0%) and 50 ng/ml bFGF (100%). Note in no cases were the fibroblasts more angiogenic than medium alone.

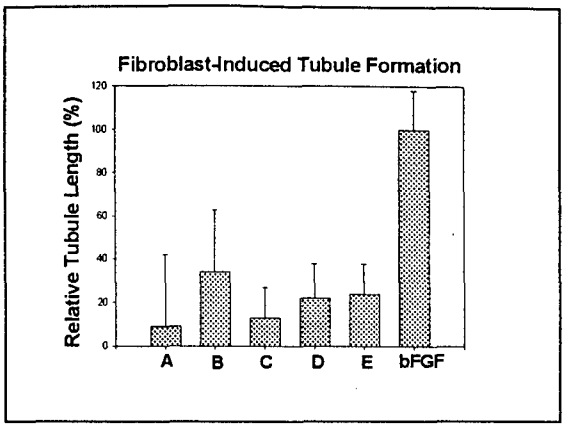


Figure 2 shows that fibroblasts from normal tissue (A and B) or tumors (C and D) were unable to inhibit endothelial tubule formation in vitro in the presence of 50 ng/ml bFGF. In all cases the degree of tubule formation was statistically indistinguishable from bFGF alone.

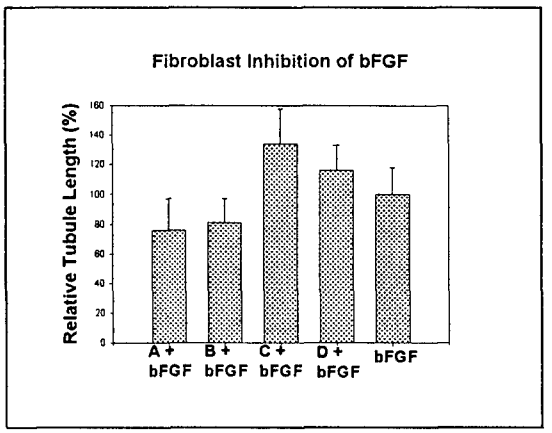


Figure 3 shows that fibroblasts from normal tissue (A and B) or tumors (C and D) were unable to inhibit endothelial tubule formation in vitro in the presence of 100 ng/ml VEGF. In all cases the degree of tubule formation was statistically indistinguishable from VEGF alone.

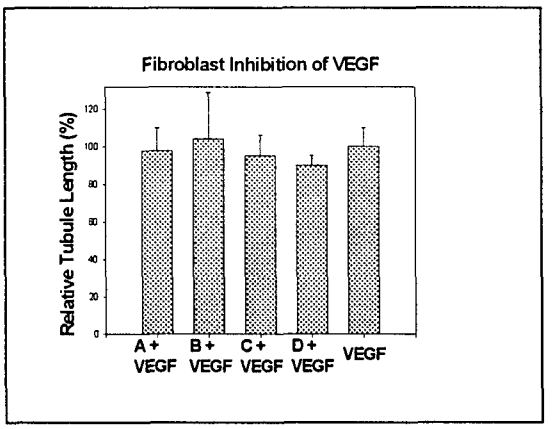


Figure 4 shows that human tumor fragments are able to induce endothelial tubules in vitro equivalent to that induced by 50 ng/ml bFGF. Skeletal muscle is not angiogenic in vitro.

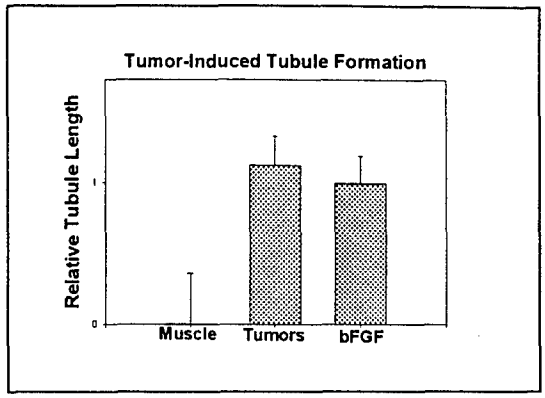
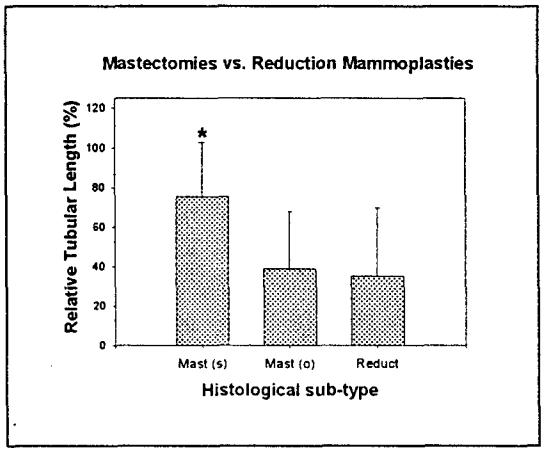


Figure 5 shows that normal epithelium isolated from mastectomy specimens in the same quadrant as the primary tumor [Mast(s)] is angiogenic in vitro whereas normal epithelium from other quadrants in the mastectomy specimen [Mast(o)] or from reduction mammoplasties [Reduct] does not show in vitro angiogenesis. * = $P<0.05$. All data are normalized to medium (0%) and 50 ng/ml bFGF (100%). Data are the mean of 10 individual patient isolates in each category.



ABBREVIATIONS

BFGF	Basic fibroblast growth factor
HUVEC	Human umbilical vein endothelial cells
Mast	Mastectomy
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide
Reduct	Reduction mammoplasty
VEGF	Vascular endothelial cell growth factor

Angiogenic Growth Factors in Preinvasive Breast Disease[1]

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ABSTRACT

Recently, we showed that preinvasive breast pathologies, such as usual hyperplasia, atypical hyperplasia, and carcinoma *in situ*, have an increased vascularity when compared with normal breast tissue (S. C. Heffelfinger *et al.*, Clinical Cancer Res., *2:* 1873–1878, 1996). To understand the mechanism of this increased vascularity, we examined by immunohistochemistry each of these pathological lesions for the expression of angiogenic growth factors. These studies showed that normal breast tissue contains numerous angiogenic agents, particularly vascular endothelial cell growth factor and basic fibroblast growth factor. At the transition from normal epithelium to proliferative breast disease, insulin-like growth factor (IGF) II expression was increased, primarily in the stroma and infiltrating leukocytes. However, among proliferative tissues, IGF I decreased with increasing vascularity. Finally, both epithelial vascular endothelial growth factor and epithelial and leukocytic platelet-derived endothelial cell growth factor increased at the transition to carcinoma *in situ*, whereas stromal and leukocytic basic fibroblast growth factor were elevated only in invasive carcinoma. Therefore, during histological progression there is also a complex progression of angiogenic growth factors. For CIS, two forms of vascularity are found: stromal microvascular density (MVD), and vascularity associated with the epithelial basement membrane (vascular score). There was 35% discordance between these two measurement systems. Among carcinoma *in situ* cases, decreases in stromal IGF II were associated with increasing vascular scores but not MVD, and increases in platelet-derived endothelial cell growth factor were associated with increasing MVD but not the vascular score. The presence of discordance and differential association with specific angiogenic agents suggests that these two forms of vascularity may be differentially regulated.

INTRODUCTION

The growth of invasive carcinoma beyond a few cubic millimeters requires the induction of a new blood supply (1). However, even before a tumor reaches the invasive state, neovascularization of the tissue may occur. We and others have shown that during breast tumorigenesis, tissue vascularity increases very early in the process of transformation, potentially before histopathological changes have occurred and certainly by the stage of usual hyperplasia (2–6). In our study of tissue vascularity in archival tissues, we also found that histologically normal epithelium was more vascular in women who had or would soon have a diagnosis of invasive breast cancer than in women who did not have invasive disease (2). Others have recently confirmed these findings *in vivo* (7). Furthermore, the vascularity of UH,[3] AH, and various grades of CIS increased with disease progression and correlated with those histological features that predict relative risk of subsequently developing invasive carcinoma (2).

Tumor vascularity is thought to be regulated by the opposing forces of angiogenic stimulators and inhibitors (8). These, in turn, are regulated, at least in part, by genetic changes resulting in tumor progression and altered local environmental conditions, such as hypoxia (9, 10). Which of the many known angiogenic regulators identified in invasive tumors may operate at the preinvasive stage of disease is completely unknown, and to date, there has not been any systematic examination of angiogenic factors in preinvasive breast pathologies. To better understand how the increase in tissue vascularity with breast tumorigenesis may be regulated, we examined archival tissues of preinvasive breast pathologies for proteins that are known to stimulate angiogenesis. Just as tissue microvascularity is of prognostic significance in invasive disease (11) the expression of angiogenic factors, such as VEGF (12, 13), bFGF (14), and PD-ECGF (15), have been associated with a worse prognosis in patients with invasive breast disease. These studies suggest that the regulation of an angiogenic phenotype may vary considerably among breast cancers. Therefore, in our examination of preinvasive breast disease, we chose to examine a broad spectrum of angiogenic growth factors. Included in this study were VEGF, bFGF, IGFs I and II (16, 17), PDGF-B (18), and PD-ECGF, which is also known as thymidine phosphorylase (19). Immunohistochemical examination of these proteins indicate that angiogenic factors are present in abundant amounts in normal breast tissue, primarily in the epithelium. With disease progression, the presence of several growth factors increases in the epithelium, as well as in other cell types. Therefore, several

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[1] These studies were supported by DAMD17-94-J-4271 from the Department of Defense.

[2] To whom requests for reprints should be addressed, at Department of Pathology, University of Cincinnati, Cincinnati, OH 45267-0529. Phone: (513) 338-1795; Fax: (513) 558-2141; E-mail: sue.heffelfinger@uc.edu.

[3] The abbreviations used are: UH, usual hyperplasia; AH, hyperplasia with atypia; CIS, carcinoma *in situ*; DCIS, ductal CIS; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; MVD, mean vascular density.

angiogenic growth factors derived from multiple cell types may be important in regulating the graded increase in tissue vascularity seen in breast tumor formation.

MATERIALS AND METHODS

Tissue Characteristics. Paraffin-embedded archival tissues from 89 patients were retrieved from the pathology files at the University of Cincinnati based on a search for specimens that contained either epithelial hyperplasia, atypical hyperplasia, or carcinoma *in situ*. Specimens included mastectomies, excisional biopsies, and reduction mammoplasties. Two observers (S. C. H. and R. Y.) independently confirmed each diagnosis based on the consensus criteria (20). Among the identified cases, 96% contained normal epithelium; 53%, UH; 12%, AH; 62%, CIS; and 29%, invasive carcinoma. Patients were seen between 1980 and 1995. The vascular scores for each tissue and patient demographics of the population have been reported previously (2).

Immunohistochemistry. All immunohistochemistry was performed on 4-μm, paraffin-embedded sections using the Ventana ES immunostaining system. After deparaffinization in xylenes and any required pretreatments, slides were placed in the instrument that adds the primary antibody, the biotinylated antimouse or rabbit secondary antibody, and avidin-conjugated peroxidase or alkaline phosphatase as dictated by a bar code. Primary antibodies were incubated for 32 min at 37°C. The instrument performed all washes. Primary antibodies were for VEGF (polyclonal A20, 1:400; Santa Cruz Biotechnology, Santa Cruz, CA), bFGF (polyclonal Ab-2, 1:50; Oncogene Research Products, Cambridge, MA), PDGF-B (N-30 anti-peptide polyclonal, 1:125; Santa Cruz Biotechnology), PD-ECGF (AB-1, PGF44, 1:200; Lab Vision Corp., Fremont, CA), IGF I and IGF II (polyclonal, 1:400; R and D Antibody, Berkeley, CA), and von Willebrand factor (polyclonal, 1:10,000; Dako, Carpenteria, CA). All tissues were trypsinized (Ventana Instruments, Tuscon, AZ) prior to addition of the primary antibody, except for those to be stained with anti-PD-ECGF. For this antibody, antigen retrieval using 0.01 M citrate buffer (pH 8.0) proved the most effective. The slides were either counterstained with hematoxylin or with nuclear fast red, by hand. In all cases, an irrelevant mouse or rabbit immunoglobulin was used instead of the primary antibody as a negative control. Two observers assessed the reaction to each antibody, recording the results using a relative intensity scale of 0–4. Intensity values were compared with an immunostained control slide containing multiple breast specimens on which examples of 0–4 staining were agreed upon. The maximal staining value for each histological tissue type was then reported in the epithelium (luminal and myoepithelium), stroma, adjacent endothelium, and leukocytes as the mean score ± SD.

Antibody Specificity. The primary antibodies (listed above) are all commercially available. Specificity of immunoreactivity was tested for each as follows. Reactivity for VEGF and IGF I and IGF II were compared on a subset of 10–20 cases to slides stained using commercial reagents from sources other than those used in the primary study. For VEGF, the additional antibodies tested were a polyclonal antibody from Biogenix (San Ramon, CA; 1:20) and a monoclonal antibody from Lab Vision (Fremont, CA; clone JH12, 1:10). Additional reagents for IGF were polyclonals from Intergen (Purchase, NY; IGF I at 1:60 and IGF II at 1:40). Because the PDGF-B antibody was antipeptide, we tested for antigen specificity by peptide inhibition of antibody reactivity *in situ*. Briefly, 5–50-fold by weight purified peptide (N-30; Santa Cruz Biotechnology) was preincubated for 2 h at room temperature with the diluted antibody prior to immunohistochemistry. Finally, antibodies to bFGF and PD-ECGF were tested by Western blot against cell extracts known to be positive for these antigens by immunohistochemistry of the cell blocks (Cytoblocks; Shandon, Pittsburgh, PA). SK-Hep1 (a gift from Gretchen Darlington; Baylor College of Medicine, Houston, TX; Ref. 21) and A431 cells (American Type Culture Collection, Rockville, MD) were grown as recommended and scraped directly into SDS-PAGE buffer (22). Aliquots were boiled, and the proteins were separated on a 4–20% acrylamide gradient gel under reducing conditions, blotted onto nitrocellulose, and tested for immunoreactivity to either bFGF or PD-ECGF using 5 μg/ml of the primary antibody. Specificity of reactivity was determined by either molecular weight analysis or comigration with purified recombinant growth factor (bFGF; Becton Dickinson, Franklin Lakes, NJ).

Vascular Score. Vessels were defined by immunohistochemistry for von Willebrand factor. Vascularity was examined by two methods. In all tissues, the vascular score was determined for each histological type of epithelium, as described previously (2). Briefly, this is a quartile score representing the proportion of basement membrane for each alveolar or ductal unit that is surrounded by vessels: 1, less than 1/3; 2, 1/3–2/3; 3, greater than 2/3 of the circumference being surrounded by vessels; and 4, complete encircling. Within a tissue, a mean vascular score is calculated from the ductal or alveolar units within each histological category. In addition to the vascular score, we determined a subjective MVD on a 0–4 scale based on observation of von Willebrand factor-stained vessels in the adjacent stroma, as defined by others (23).

Statistics. Mean immunoreactive scores among groups were compared by ANOVA on ranks and Tukey test (SigmaStat; SPSS Science, Chicago, IL). Data for the percentage of positive cases were compared by Z test (SigmaStat, SPSS Science).

RESULTS

Specificity of Immunoreagents and Concordance in Interpretation. Each primary antibody was tested for specific immunoreactivity by either comparing the staining pattern with commercial antibodies of the same specificity in a subset of cases (VEGF, IGF I, and IGF II), inhibiting immunoreactivity with excess peptide immunogen (PDGF-B), or Western blot analysis of cell extracts (bFGF and PD-ECGF). Fig. 1 shows the results of these studies, which support the antigen specificity of these commercial reagents.

Fig. 2 shows an example of immunostaining intensity values ranging from negative (0) to extremely intense (4). Staining concordance was determined in 10% of cases for each antibody. Interobserver variability in determining stain intensity of the same cells (*i.e.*, performed by two readers independently while sitting at a multiheaded microscope) was 86% for all antibodies, varying from 78–96%. The highest variability was during the

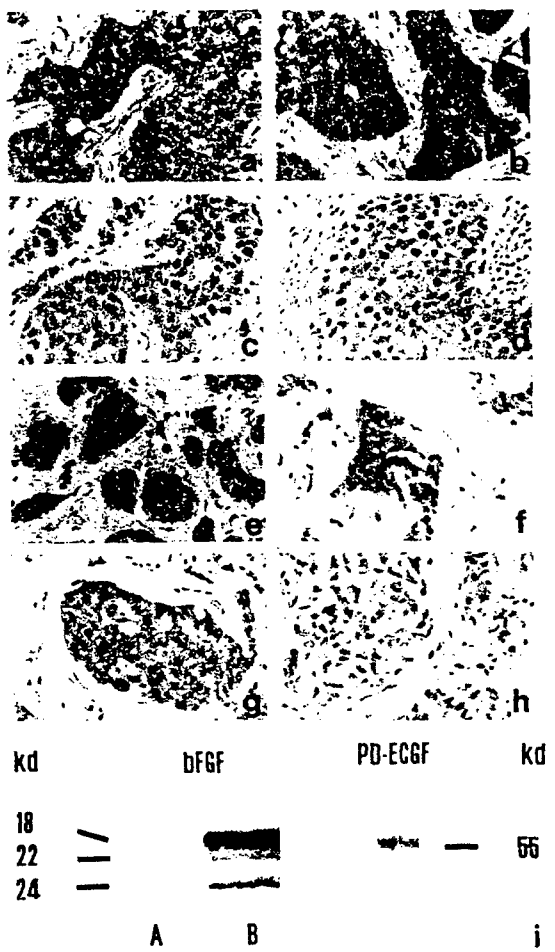

Fig. 1 *a* and *b*, immunoreactivity to VEGF. *a*, polyclonal antibody from Santa Cruz Biotechnology used for our primary data; *b*, polyclonal antibody from Biogenix with the same staining pattern as in *a*. A monoclonal antibody from Lab Vision also gave similar results (data not shown). *c–f*, IGF I and IGF II immunoreactivity with polyclonal antibodies from R&D Antibody, used for our primary data (*c* and *e*) and from Intergen (*d* and *f*), respectively. Both of these sets of reagents showed the same reactivity on multiple specimens. Peptide inhibition of PDGF-B immunoreactivity is illustrated in *g* (without peptide) and *h* (with 5-fold excess peptide). *a–h*, same magnification (×132). Western blot analysis of cell extracts is shown for antibodies against bFGF and PD-ECGF (*i*). Because bFGF is known to have multiple molecular weight products of M_r 18,000, 24,000, and 27,000 (53), we confirmed the multiple molecular weight bands identified in our SK hep-1 cell extracts by examining recombinant protein on the same gel. Ten-fold excess recombinant bFGF diminished staining of these bands (data not shown). Western analysis of A431 extracts showed the expected M_r 55,000 band for PD-ECGF.

discrimination of intensities as either 3 or 4 and therefore was the poorest for bFGF. On the other hand, weak staining was rarely disputed, showing the best concordance for PDGF and IGF I. In no case did either intraobserver or interobserver values differ in intensity by more than 1. Intraobserver variability was determined by an individual rereading cases at an interval of several months; therefore, these data test not only consistency in determining intensity scale but also selecting the most intensely stained cell(s) within each histological tissue type for each

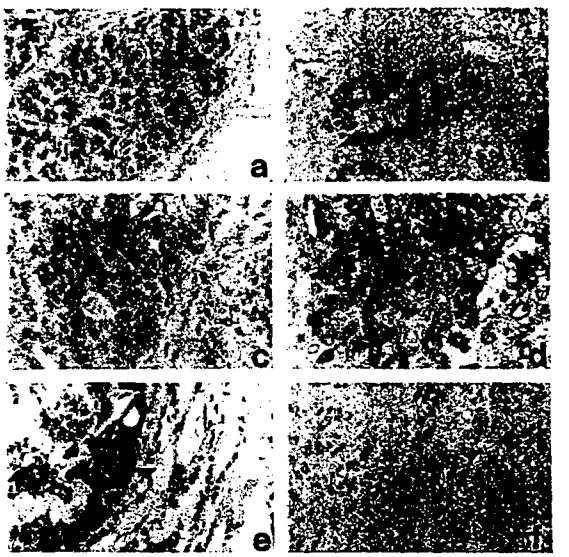

Fig. 2 Immunohistochemistry for PD-ECGF showing the range of staining intensity (in parentheses) among histological subtypes of epithelium. *a*, normal tissue with no immunoreactivity (0). *b*, proliferative epithelium with minimal staining (1). *c*, atypical hyperplasia with low staining (2). *d*, CIS with significant staining (3). *e*, invasive disease with intense staining (4). *f*, invasive disease stained with an isotype specific mouse immunoglobulin of unrelated specificity (0). Note that the intensity scale is based on the most intense cytoplasmic staining within the histological subtype of epithelium (*arrowheads*). All photographs are of the same magnification (×132).

antibody tested. Mean concordance within one intensity value for all antibodies was 84%.

Epithelial VEGF Increases with Progression. Table 1 shows the mean relative intensity and percentage of cases positive for VEGF immunoreactivity at all stages of progression in all cases tested. Table 1 also shows the mean immunoreactivity for VEGF, excluding negative cases. The relative intensity of VEGF immunoreactivity in the epithelium increased with progression to invasion, being statistically greater in CIS and invasion than in normal epithelium. VEGF staining of invasive epithelium was also statistically greater than that of UH. VEGF staining was present to some extent in nearly all types of epithelium, indicating that the increase in mean relative intensity is attributable to a true increase in immunoreactivity per cell type with progression and not simply due to an increase in the number of cases showing immunoreactivity. Stromal mean staining intensity for VEGF was considerably lower than in the epithelium, and although there was a trend for stromal staining to increase with progression, this was not statistically significant. This lower mean staining intensity was due both to less staining of individual cases in the stroma relative to the epithelium and fewer positive cases. There was no change in vascular or leukocyte VEGF immunoreactivity with progression, with relative intensity means of 1.61 and 1.08, respectively; staining was fairly ubiquitous in the endothelium, whereas it was identified in leukocytes in only a one-third to one-half of cases.

bFGF Increases in Nonepithelial Cell Types in Invasive Disease. Table 2 shows the mean relative intensity of immunoreactivity for bFGF and the percentage of positive cases for

Table 1 VEGF immunoreactivity

	Mean immunoreactivity in all cases by histological type[a]		Cases that are VEGF immunoreactive[b]				Mean VEGF immunoreactivity among positive cases[c]	
	Epi[d]	Str	Epi	Str	Endo	WBC	Epi	Str
NL	2.20 ± 1.03	0.55 ± 0.67	96	49	86	41	2.26 ± 0.98	1.17 ± 0.45
UH	2.33 ± 0.72	0.51 ± 0.63	100	43	81	33	2.33 ± 0.72	1.15 ± 0.37
AH	2.67 ± 0.50	0.56 ± 0.53	100	56	89	33	2.67 ± 0.50	1.00 ± 0.00
CIS	2.75 ± 0.79[e]	0.87 ± 0.88	100	60	83	47	2.75 ± 0.79[e]	1.46 ± 0.64
Inv	3.19 ± 0.63[e]	0.85 ± 0.97	100	54	85	54	3.19 ± 0.63[f]	1.57 ± 0.76

[a] Mean relative staining intensity ± SD.
[b] Percentage of positive cases showing VEGF immunoreactivity for each cell type.
[c] Mean relative staining intensity ± SD among cases that are positive for VEGF immunoreactivity.
[d] Epi, epithelium, Str, stroma, Endo, endothelium, NL, normal cells, Inv, invasive carcinoma.
[e] Statistically greater than values for normal epithelium; $P < 0.001$.
[f] Statistically greater than values for UH; $P < 0.001$.
[g] Mean relative staining intensity ± SD.

Table 2 bFGF immunoreactivity

	Mean bFGF immunoreactivity in all cases by histological type[a]			% positive cases that are bFGF immunoreactive[b]			
	Epi	Str	WBC	Epi	Str	Endo	WBC
NL	2.79 ± 0.80	0.77 ± 0.87	1.19 ± 1.46	100	55	89	42
UH	2.92 ± 0.75	1.09 ± 0.99	1.13 ± 1.48	98	66	81	40
AH	2.73 ± 0.79	0.91 ± 0.30	0.91 ± 1.3	100	91	100	36
CIS	2.58 ± 1.03	1.20 ± 0.95	1.51 ± 1.50	93	73[d]	84	55
Inv	2.97 ± 0.68	1.45 ± 1.21[c]	2.28 ± 1.51[c]	100	76	93	72[e]

[a] Mean relative staining intensity ± SD.
[b] Percentage of positive cases showing bFGF immunoreactivity in each cell type.
[c] Statistically greater than values for normal epithelium; $P < 0.001$.
[d] $P = 0.049$.
[e] $P = 0.01$, compared with the values in normal tissue.

each histological cell type during progression. Epithelial immunoreactivity was ubiquitous at a fairly high level with no changes during progression. On the other hand, stromal bFGF, although being less intense than epithelial staining, increased during progression and was statistically higher in invasive disease relative to stroma in normal tissue. However, this increase is due almost entirely to the increase in percentage of positive cases with progression. Although endothelial staining was fairly ubiquitous, the mean relative intensity was quite low (~1.0) throughout progression. However, leukocyte bFGF increased during progression, being statistically higher in invasion relative to normal tissue or UH. Again, this was attributable to the large influx of inflammatory cells in invasive tumors and not a change in relative staining intensity of individual cells at different stages of progression.

PDGF-B Expression Is Constant during Progression. PDGF- B immunoreactivity was found primarily in the epithelium with low level staining in the endothelium and stroma and essentially no staining of leukocytes. Table 3 shows the mean relative level of immunoreactivity in the epithelium, endothelium, and stroma for all cases. Although there appears to be an increase in endothelial staining in invasive disease, it is not statistically different from the other stages of progression and is due entirely to the increase in the percentage of positive cases.

Epithelial and Leukocytic PD-ECGF Increases Significantly at the Stage of CIS. PD-ECGF staining was identified essentially only in the epithelium and in leukocytes. Mean levels of immunoreactivity for all cases were higher in CIS and invasive disease in both of these cell types relative to normal tissue or UH, as shown in Table 4. The increase with progression in epithelial staining is accounted for entirely by the increase in the percentage of positive cases with progression (Table 4). However, the increase in leukocyte mean reactivity is due to both an increase in staining intensity per cell and the influx of leukocytes with progression (Table 4).

IGF II Increases Early in Progression, Whereas IGF I Remains Constant. Table 5 shows mean IGF I and IGF II immunoreactivity, percentage of positive cases, and mean immunoreactivity, excluding negative cases. Epithelial IGF I immunoreactivity was found in roughly one-half of the cases, with a slight increase in mean staining intensity with progression. This increase was entirely due to an increase in the percentage of positive cases. IGF II, on the other hand, was much more prevalent in the epithelium. IGF II also showed slight increases with progression, but in this case, the increase was attributable to more intense staining within individual cases with progression. Endothelial immunoreactivity for IGF I was rare; IGF II, however, was identified on the endothelium in between a one-

Table 3 PDGF-B immunoreactivity

	Mean PDGF-B immunoreactivity in all cases by histological type[a]			% positive cases that are PDGF-B immunoreactive			
	Epi	Endo	Str	Epi	Str	Endo	WBC
NL	1.05 ± 0.86	0.20 ± 0.53	0.18 ± 0.56	74	14	15	1
UH	1.37 ± 0.97	0.20 ± 0.45	0.13 ± 0.34	83	13	17	2
AH	1.58 ± 1.16	0.08 ± 0.29	0.17 ± 0.39	83	17	8	0
CIS	1.24 ± 1.09	0.20 ± 0.56	0.07 ± 0.26	69	9	27	4
Inv	1.07 ± 1.09	0.46 ± 0.74	0.26 ± 0.07	57	7	32	4

[a] Mean relative staining intensity ± SD.
[b] Percentage of positive cases showing PDGF-B immunoreactivity.

Table 4 PD-ECGF immunoreactivity

	Mean PD-ECGF immunoreactivity in all cases by histological type[a]		% positive cases that are PD-ECGF immunoreactive[b]				Mean PD-ECGF immunoreactivity among positive cases[c]	
	Epi	WBC	Epi	Str	Endo	WBC	Epi	WBC
NL	0.78 ± 1.02	0.94 ± 1.37	49	1	8	35	1.58 ± 0.92	2.67 ± 0.83
UH	1.25 ± 0.96	1.60 ± 1.57	67	2	13	56[h]	1.58 ± 0.79	2.85 ± 0.86
AH	1.17 ± 1.17	1.83 ± 1.60	67	0	0	67	1.75 ± 0.98	2.75 ± 0.96
CIS	1.82 ± 1.22[d]	2.62 ± 1.50[e]	85[f]	4	9	82[i]	2.13 ± 1.03	3.20 ± 0.92
Inv	1.48 ± 1.22[d]	3.22 ± 1.31[e]	75[g]	7	4	89[j]	2.00 ± 0.97	3.63 ± 0.65[e]

[a] Mean relative staining intensity ± SD.
[b] Percentage of positive cases showing PD-ECGF immunoreactivity.
[c] Mean relative staining intensity ± SD among cases that are positive for PD-ECGF.
[d] Statistically greater than values for normal epithelium; $P < 0.001$.
[e] Statistically greater than values for normal epithelium and UH; $P < 0.001$.
[f] $P < 0.001$, relative to normal epithelium.
[g] $P = 0.043$, relative to normal epithelium.
[h] $P = 0.034$, relative to normal tissue.
[i] $P = 0.008$, relative to UH, and $P < 0.001$, relative to normal tissue.
[j] $P = 0.007$, relative to UH, and $P < 0.001$, relative to normal tissue.

third and one-half of cases, independent of histology (Table 5). Among those cases positive for endothelial IGF II staining, the relative intensity varied from 1.7 to 2.0. Stromal staining for both IGF I and IGF II was found in one-third to one-half of cases, independent of histology. Similar to the epithelium, IGF II also increased in stain intensity during progression in the stroma, whereas with IGF I, the stromal staining was fairly constant. Finally, leukocyte staining was identified in a similar number of cases for both IGF I and IGF II. However, stain intensity was constant for IGF I and increased for IGF II.

Growth Factor Expression, Vascularity, and the Presence of Invasion. Because we had shown previously that normal breast tissue from women with invasive disease was more vascular than tissue from women without invasive disease (2), we compared the presence of each angiogenic factor with immunoreactivity in normal epithelium and stroma between these two populations. No correlation between invasive disease and the presence of individual angiogenic factors in normal epithelium or stroma was identified. Similarly, there was no correlation for any of the growth factors between epithelial or stromal staining and the level of vascularity in normal tissue.

As noted above, only IGF II showed significant changes early in progression, with significant increases being found in stromal and leukocyte staining. IGF I staining remained essentially stable in these same tissues. Therefore, we compared IGF I and IGF II staining with the degree of vascularity among all proliferative specimens. Surprisingly, both epithelial and stromal IGF I immunoreactivity was decreased in those proliferative specimens with the greatest vascularity (vascular score of >1 *versus* <1, with the range of vascular scores being 0–1.67). As shown in Fig. 3*a*, this was significant ($P = 0.021$) in the epithelium. IGF II, on the other hand, showed slight to no decrease with increased vascularity (Fig. 3*b*). None of the other angiogenic factors showed any change with vascularity among the proliferative lesions.

Because most of the changes in angiogenic factor expression were found among the carcinomas *in situ*, we compared these data with the degree of vascularity. Vascularity was assessed as we have reported previously (2), basically restricting the quantification to those vessels that actually touch the epithelial basement membrane. In addition, we assessed the microvascular density in the adjacent connective tissue, as has been reported by others (23). The first we refer to as the "vascular score," and the second, as MVD. Thirty-five % of cases showed discordance between these methods, *i.e.*, were high by one method *versus* low in the other. However, as shown in Fig. 4, both mechanisms of assessment showed statistically significant differences between non-comedo ductal CIS and comedo carcinoma. Epithelial VEGF, bFGF, PD-ECGF, and IGF II immunoreactivity were compared against vascular score and MVD

Table 5 IGF I and II immunoreactivity

	Mean IGF I and II immunoreactivity in all cases by histological type[a]					
	IGF-I			IGF-II		
	Epi	Str	WBC	Epi	Str	WBC
NL	0.85 ± 0.87	0.46 ± 0.69	0.41 ± 0.87	1.89 ± 1.17	0.48 ± 0.67	0.59 ± 1.11
UH	1.13 ± 1.00	0.44 ± 0.65	0.26 ± 0.77	2.15 ± 1.22	0.58 ± 0.85	0.42 ± 0.96
AH	0.69 ± 0.75[d]	0.23 ± 0.83	0.15 ± 0.55	1.85 ± 1.34	0.31 ± 0.85	0.15 ± 0.55
CIS	1.34 ± 1.11[d]	0.55 ± 0.75	0.30 ± 0.80	2.42 ± 1.42	0.64 ± 0.91	0.62 ± 1.21
Inv	1.21 ± 1.29	0.68 ± 0.90	0.39 ± 1.03	2.61 ± 1.26	0.89 ± 1.07	0.50 ± 1.11

	% positive cases that are IGF-I and II immunoreactive[b]							
	IGF-I				IGF-II			
	Epi	Str	Endo	WBC	Epi	Str	Endo	WBC
NL	59	36	8	20	83	39	38	24
UH/AH	69	35	15	11	85	40	40	17
CIS	72	42	0	25	78	45	53	25
Inv	61	46	18	18	89	54	46	18

	Mean IGF I and IGF II immunoreactivity among positive cases[c]					
	IGF-I			IGF-II		
	Epi	Str	WBC	Epi	Str	WBC
NL	1.44 ± 0.65	0.48 ± 0.67	0.59 ± 1.11	2.29 ± 0.86	0.58 ± 0.70	0.71 ± 1.19
UH	1.64 ± 0.78	0.58 ± 0.85	0.42 ± 0.96	2.51 ± 0.90	1.47 ± 0.70[f]	2.50 ± 0.53[f]
AH	1.29 ± 0.49	0.31 ± 0.85	0.15 ± 0.55	2.67 ± 0.50[e]	2.00 ± 1.41	2.00 ± 1.00
CIS	1.69 ± 0.98	0.64 ± 0.91	0.62 ± 1.21	3.03 ± 0.82[e]	1.40 ± 0.87[f]	2.83 ± 0.58[f]
Inv	2.00 ± 1.06	0.89 ± 1.07	0.50 ± 1.11	2.92 ± 0.91[f]	1.67 ± 0.90[f]	2.80 ± 0.45[f]

[a] Mean relative staining intensity ± SD.
[b] Percentage of positive cases showing IGF I or II immunoreactivity.
[c] Mean relative staining intensity ± SD among cases that are positive for IGF I or IGF II.
[d] Statistically greater than values for normal epithelium, $P = 0.042$.
[e] Statistically greater than values for normal epithelium and UH; $P < 0.001$.
[f] Statistically greater than values for normal epithelium; $P < 0.001$.

among all CIS. No differences were detected using vascular score (Fig. 5*a*). However, PD-ECGF was significantly higher in those cases of DCIS with the highest MVD (Fig. 5*b*). Although comedo DCIS is more vascular than non-comedo DCIS, the difference in PD-ECGF among the more vascular tumors was not attributable to this difference, because PD-ECGF expression was not statistically greater in comedo than in non-comedo carcinomas (data not shown). On the other hand, when examining stromal staining for these growth factors, we found that no changes were seen among angiogenic factors relative to MVD (Fig. 6b), but that IGF II was significantly decreased with increases in vascular score (Fig. 6*a*).

DISCUSSION

Increases in tissue vascularity are attributable to numerous processes including tissue remodeling, angiogenesis, and vasculogenesis (24). The contribution of each of these processes and their regulation in invasive cancer are still poorly understood. Even less well-understood are the mechanisms of increased tissue vascularity in preinvasive tumor formation.

We identified an increase in tissue vascularity in preinvasive breast pathologies that correlated with histological features of disease progression (2). In this study, we surveyed growth factors known to be angiogenic *in vitro* and *in vivo* to begin looking at mechanisms of vascular growth regulation during the process of tumorigenesis. Surprisingly, even normal breast tissue is awash in angiogenic proteins, independent of the presence of progressive disease. Therefore, the mere presence of angiogenic growth factors is insufficient to initiate an angiogenic phenotype. This phenomenon has also been seen by others and is thought to be attributable to the high level of angiogenic inhibitors in normal tissues (25). To date, we have not examined these tissues for angiogenic inhibitors. However, it is clear that during progression, the expression of numerous angiogenic agents increases, and these agents can be found in many cell types. Because we confined these studies to immunohistochemical stains, we cannot determine the cell of origin for each angiogenic factor; however, the presence of the protein in the tissue is clearly significant. Furthermore, because multiple histological stages are present on a single slide, we can directly compare changes in expression within individual cases and among different cell types.

Three of the growth factors examined (VEGF, bFGF, and PD-ECGF) showed significant increases at the transition from AH to CIS. Generally, these elevated levels were maintained in the invasive component of the tumor. VEGF showed a continuous slow increase with progression, which was only statistically significant at the stage of CIS. Clearly, VEGF expression

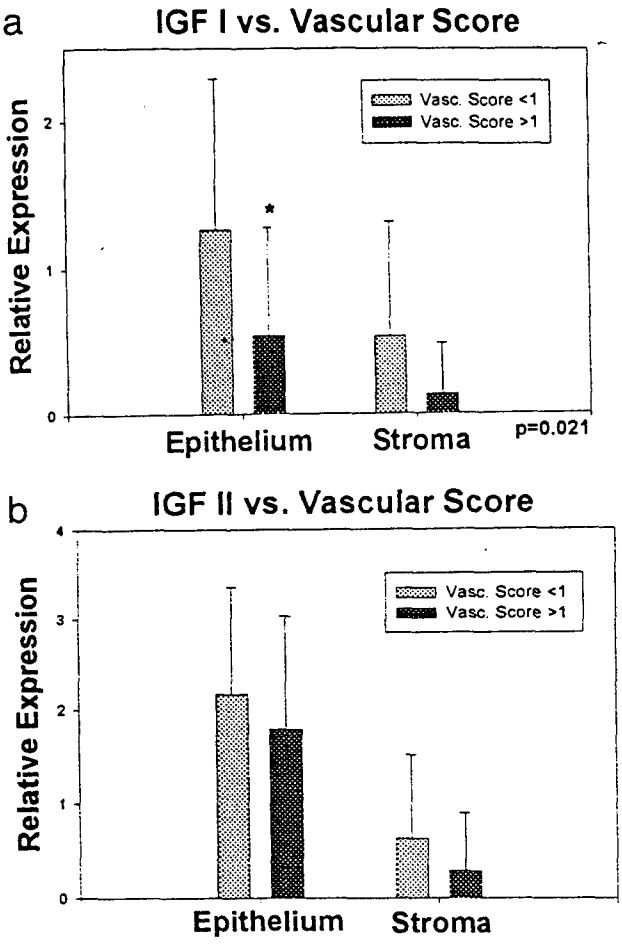

Fig. 3 Shown are the mean expression (*bars*, SD) of IGF I (*a*) and IGF II (*b*) in proliferative tissues with vascular scores of <1 or >1. Note the decrease in IGF I expression in the epithelium with the highest vascular score. *, $P = 0.021$.

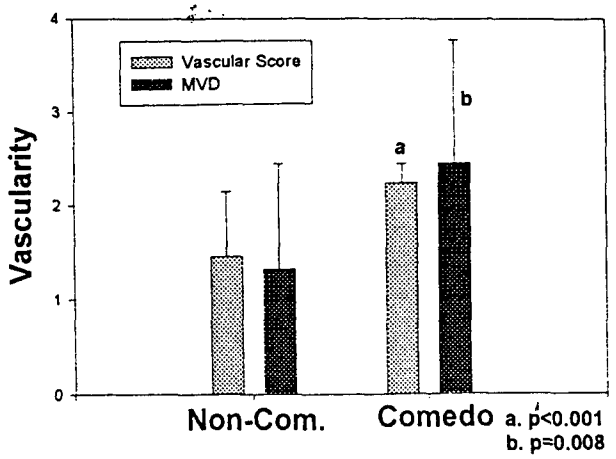

Fig. 4 Mean vascular score and MVD (on the same scale; *bars*, SD) for non-comedo and comedo DCIS. Vascularity determined by either method is higher is comedo CIS than in non-comedo CIS.

is not suddenly turned on at this level of progression. Interestingly, VEGF in particular has been thought to mediate significant changes in angiogenesis with small changes in expression (26). The mean increase in expression in VEGF from normal tissue to invasion is on the order of 25% (assuming a linear scale). If true during breast tumorigenesis, then VEGF may play a significant role in regulating the marked up-regulation of vascularity seen at the stage of CIS. Numerous groups have examined VEGF expression in CIS and invasive breast tumors. For instance, Anan *et al.* (27) tested breast tumors and adjacent nontumorous tissues for VEGF expression by reverse transcription-PCR. They found VEGF message in only 17% of nontumorous tissues, whereas we found fairly ubiquitous protein expression. Brown *et al.* (28) also found high expression of VEGF message by *in situ* hybridization in comedo DCIS and invasive and metastatic cancers. Similarly, Toi *et al.* (29) showed that VEGF protein was predominately in the tumor cytoplasm. As in our study, they found low levels of expression in normal tissues. Guidi *et al.* (30)also found high VEGF expression in DCIS. Therefore, we and others agree that VEGF increases in the epithelium at the time of DCIS formation. Although in most cases the level of VEGF in invasive tumors correlates with microvascular density (27, 30, 31), Guidi *et al.* (30) also found some correlation between VEGF expression and microvascular density of DCIS. We found no such correlation in our tissues.

Unlike VEGF, which was primarily epithelial, bFGF and PD-ECGF increases were seen in nonepithelial components, particularly in tissues with either CIS or invasive disease. Even normal epithelium stained intensely for bFGF, but the changes during progression were in the stroma and leukocytes. This is consistent with the fact that bFGF is known to be sequestered in the stroma (32). Potentially, these growth factors are available for stimulation of endothelium upon the local release of proteolytic enzymes. Anan *et al.* (30) also measured bFGF in nontumorous breast tissue (30). They examined tissues by reverse transcription-PCR and found that bFGF message was present in the majority of both tumor and nontumor specimens, consistent with what we report here. Both Colomer *et al.* (33) and Gomm *et al.* (34) also showed the expression of bFGF in normal breast tissue. In addition, de Jong *et al.* (35) showed that in breast tumors, bFGF expression correlated with microvascular density. We examined bFGF expression in our cases of CIS and saw no correlation with MVD or vascular score.

PD-ECGF is thought to be involved in vascular remodeling during wound healing (36). In our study, the total load of PD-ECGF was so tightly related to the intensity of leukocyte infiltration that it seems clear that leukocytic induction of angiogenesis may, in a subset of cases, be similar in mechanism to inflammatory states. Moghaddam *et al.* (35) found that PD-ECGF levels were increased in breast tumors relative to normal breast tissue (35). Toi *et al.* (37) examined PD-ECGF immunostaining in invasive carcinomas and found that the presence of this growth factor correlated with microvascular density. Engels *et al.* (38) also examined PD-ECGF in breast pathologies, looking primarily at DCIS. They found no correlation with total microvascular density but did identify some correlation with the presence of a dense vascular rim around the DCIS. As in our studies, they saw only weak staining for normal breast epithe-

ization and immunohistochemistry, that IGF II is primarily in tumorous stroma and only rarely in the epithelium. However, reports by others show both IGF I and IGF II in the epithelial and stromal compartments (44–48), consistent with the hypothesis that these growth factors may function via paracrine growth regulation (49, 50). Our data indicate that IGF II increases early in progression. However, equally significant is the observation that total IGF I does not change with progression, but among proliferative lesions, IGF I decreases as vascularity increases. Together, these data suggest that the ratio of IGF I:IGF II may be important.

Our data regarding PDGF-B is consistent with other reports in that it is expressed primarily in the epithelium in both normal and malignant breast epithelium (51, 52). Coltrera *et al.* (51) reported that expression of PDGF-B correlated with cell proliferation. Our data indicate that in breast tumor progression, PDGF-B has no role in tissue vascularity.

As reported previously (2), vascular score increases with disease progression, indicating that vascularity immediately adjacent to the epithelial basement membrane occurs very early. At the level of CIS, increases in vascularity of the adjacent stroma are also clearly present in a subset cases. Although this aspect of vascularity correlates generally with vascular score, there are numerous cases of discordance, indicating that vessel growth in these two regions may be differentially (or temporally) regulated. For instance, our data show that PD-ECGF correlates with MVD but not vascular score. On the other hand, stromal IGF II inversely correlates with vascular score. Additional studies of growth factor expression in CIS are needed to resolve how these two aspects of vascularity are regulated.

In summary, just as the process of disease formation in breast tissue is complex, both morphologically and genetically, so the regulation of vascularity is multifactorial. Clearly, local conditions regulating stromal and immune reactions play some role. The genetic pathways of epithelial transformation must also be considered. Our data provide a groundwork on which to examine the interactions of these mechanisms with tissue vascularity. Knowledge of the regulatory processes that control vascularity in these tissues may provide targets for chemopreventive strategies in women with preinvasive breast pathologies.

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Running title: CYCLIN D AND VASCULARITY

Cyclin D1, Retinoblastoma, p53, and Her2/neu Protein Expression in Pre-invasive Breast Pathologies: Correlation with Vascularity

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Abstract

Pre-invasive breast pathologies show a degree of vascularization that correlates with risk of invasion. Recently, numerous oncogenes and tumor suppressor genes have been shown to regulate neovascularization. Therefore, we examined by immunohistochemistry archival tissues of pre-invasive breast pathologies for alterations in the expression of three genes, cyclin D1, retinoblastoma (Rb), p53, and Her2/neu, known to be important in breast tumorigenesis, and correlated these data with tissue vascularity. Vascularity was determined by immunologic detection of von Willebrand factor. For CIS both stromal vascularity (MVD) and vascular cuffing (MCD) were determined. We found that cyclin D1 expression was increased in usual hyperplasia (11% of cases). Atypical hyperplasia, non-comedo CIS and comedo CIS were positive in 43%, 49%, and 57% of cases, respectively. Increased expression was also found in lobular CIS. Changes in Rb and p53 were rare in hyperplasia but occurred in 8% and 10% of CIS, respectively. Her2/neu protein was identified rarely in atypical hyperplasia and in both non-comedo and comedo ductal CIS. Neither Rb nor Her2/neu expression correlated with vascularity. P53 immunoreactivity correlated positively with both MCD and MVD. Cyclin D1 was negatively associated with MVD. These data suggest that genetic alterations may regulate the angiogenic phenotype.

Key words: Breast cancer, angiogenesis, Her2/neu, cyclin D1, retinoblastoma, p53

Introduction:

In many women the development of invasive breast cancer is presaged by breast pathologies that indicate the presence of genetic damage. These pre-invasive lesions, such as usual hyperplasia (UH), atypical hyperplasia (AH), and carcinoma in situ (CIS), may in some cases be direct precursors of invasive disease. Although there have been recent advances in unraveling the genetic changes associated with breast tumorigenesis, we still have little understanding of what mutations are likely to be early events. Genes that are known to be involved in pre-invasive pathologies include Her2/neu, EGFR, p53, and various cyclins (Gusterson et.al.1988; Van de Vijver et. al.1988; Iglehart et. al. 1990; Liu et. al. 1992; De Pottier et. al. 1994; Contegiacomo et. al.1995; Klijn et. al.1992; von Agthoven et. al. 1994; Scala et. al.1995; Walker et. al. 1991; Elledge and Allred 1994; Trudel et. al. 1992; Pietilainen et. al. 1995; Weinstat-Saslow et. al. 1995; Zukerburg et. al. 1995; Jares et. al. 1997; Alle et. al. 1998;). In addition, there are numerous less defined chromosomal abnormalities (Gillett et. al. 1998; Stanta et. al. 1998; Chen et. al. 1991; Mars and Saunder 1990; Andersen et. al. 1992; Micale et. al. 1994; O'Connell et. al. 1994; Balazs et. al. 1995; Murphy et. al. 1995; Radford et. al. 1995; Ried et. al. 1995). Studies in which a large number of genomic sequences have been examined consistently show that CIS is a genetically advanced state often with abnormalities identical to adjacent invasive disease (Visscher et. al. 1993; Zuang et. al. 1995). On the other end of the

spectrum, UH and even normal epithelium have in some cases been found to harbor genetic damage (Dietrich et. al. 1995; Deng et. al. 1996; Lakhani et. al. 1996; Kasami et. al. 1997; Larson et. al. 1998). According to the stochastic model of tumorigenesis, a series of genetic changes which result in required phenotypic manifestations may be accumulated in any temporal sequence, that is mutations resulting in increased proliferation, motility, or angiogenesis may occur in any order, but all are required for the fully transformed state (Smith 1990; Smith et. al. 1993). However, we now know that because of the pleotropic signaling events regulated by many oncogenes and tumor suppressor genes, several phenotypic events may be altered by a single genetic event.

Angiogenesis is one phenotype that is expressed very early in the transformation process in a number of model systems (Folkman et. al. 1989; Kandel et. al. 1991; Smith-McCune and Weidner 1994) and may in fact be regulated by different genetic events during tumorigenesis (Kerbel et. al. 1998; Petit et. al. 1997). Indeed, during breast tumorigenesis, we have shown that induction of an increased vasculature occurs very early in the process, at the time of epithelial hyperplasia, and additional increments in vascularity occur with disease progression (Heffelfinger et. al. 1996). Unfortunately, the genetic/epigenetic events, which underlie this phenotypic progression in breast tumorigenesis, are unknown. Recent studies indicate that a number of oncogenes and tumor

suppressor genes regulate angiogenesis (Kerbel et. al. 1998). Among those studied, many are thought to play a role in breast cancer, such as Her2/neu, epidermal growth factor receptor, ras, src, p53, and Rb (Rastinejad et. al. 1989; Dawson et. al. 1995; Mukhopadhyay et. al. 1995; Vermeulen et. al. 1996; Takahashi et. al. 1998; Dameron et. al. 1994). Several oncogenes have been shown to up-regulate vascular endothelial growth factor (VEGF), a potent angiogenic agent (Kerbel et. al. 1998). The tumor suppressor genes, Rb and p53, have both been mechanistically connected to angiogenesis either through the production of VEGF or the loss of angiogenic inhibitors (Kieser et. al. 1994; Van Meir et. al. 1994; Fontanini et. al. 1997; Grant et. al. 1998; Uchida et. al. 1998; Bouvet et. al. 1998). Indeed, it has been proposed that p53 gene therapy may inhibit tumor growth in part by inhibition of angiogenesis (Bouvet et. al. 1998).

In order to define early genetic changes in breast tumorigenesis, we examined pre-invasive breast pathologies from archival specimens for alterations in protein expression from four genes and correlated these data with tissue vascularity. Three of the genes we studied, p53, Rb, and Her2/neu, were chosen because they are implicated in regulating angiogenesis in invasive tumors, and they are known to be mutated in some pre-invasive breast pathologies (Walker et. al. 1991; Elledge et. al. 1994; Trudel et. al. 1992; Pietilainen et. al. 1995; Petit et.al. 1997). The fourth gene we studied, cyclin D1, to our knowledge, has not been directly

associated with angiogenesis by a known mechanism. However, its frequent mutation in carcinoma in situ encouraged us to examine its role in angiogenesis in pre-invasive breast pathologies (Weinstat-Saslow et. al. 1995; Zukerburg et. al. 1995; Jares et. al. 1997; Alle et. al. 1998; Gillett et. al. 1998). Furthermore, two lines of evidence implicate cyclin D1 in hormone-induced breast epithelial hyperplasia, an important component of pre-invasive breast pathologies. First, the mouse mammary tumor virus-driven cyclin D1 gene induces mammary hyperplasia and pregnancy-dependent adenocarcinomas (Wang et. al. 1994). Second, the cyclin D1 knock out mice lack pregnancy-induced mammary proliferation (Sicinski et. al. 1995). Therefore, cyclin D1 may be important in human epithelial hyperplasias.

To determine whether identified alterations in protein expression for these four genes may be responsible, in part, for neovascularization of pre-invasive pathologies, the immunohistochemical data were tested for correlations to tissue vascularity. Results from these studies suggest that events leading to alterations in expression of oncogenes and tumor suppression genes may alter the vascularity of pre-invasive breast disease.

Materials and Methods:

Tissue characteristics: Paraffin-embedded archival tissues from 89 patients were retrieved from the pathology files at the University of Cincinnati based on a search for specimens which contained either epithelial hyperplasia, atypical hyperplasia or carcinoma in situ. Specimens included mastectomies, excisional biopsies, and reduction mammoplasties. Two observers (SCH and RY) independently confirmed each diagnosis based on the consensus criteria (Hutter 1985). The diagnosis of atypical hyperplasia accounted for 13% of all cases with epithelial hyperplasia. Among cases with CIS, 12% were comedo, 5% were lobular, and the remainder were ductal of a non-comedo type. Patients were seen between 1980 and 1995. The patient demographics of this population have been previously reported (Heffelfinger et. al. 1996). These studies were performed following approval from of The University of Cincinnati Institutional Review Board.

Immunohistochemistry: All immunohistochemistry was performed on 4 μm, paraffin-embedded sections using the Ventana ES immunostaining system. Following deparaffinization in xylenes and any required pre-treatments, slides were placed in the instrument, which adds the primary antibody, the biotinylated anti-mouse or rabbit secondary antibody, and avidin-conjugated peroxidase or alkaline phosphatase as dictated by a bar code. Primary antibodies were incubated

for 32 minutes at 37° C. The instrument performed all washes. Primary antibodies were for cyclin D1 (P2D11F11, 1:15, Novocastra/Vector, Burlingame, California), Rb (84-B3-1, 1:100, Novocastra/Vector, Burlingame, California), p53 (DO-1, 1:300, Oncogene Research, Cambridge, MA), Her2/neu (Mab-1 and Pab-1, Zymed, South San Francisco, California) mixed according to the method of Paik et al. (1991) or CB11, and von Willebrand factor (polyclonal, 1:10,000, Dako, Carpenteria, CA). Cyclin D1 and p53 slides required antigen retrieval (microwave 20 minutes in 0.01M citrate buffer, pH 6.0). Rb slides were trypsinized (Ventana Instruments, Tuscon, AR) prior to addition of the primary antibody. The slides were either counterstained with hematoxylin or with nuclear fast red, by hand. In all cases an irrelevant mouse or rabbit immunoglobulin was used instead of the primary antibody as a negative control. Two observers (SCH and RY or SCH and EEL) assessed the reaction to each antibody. Cyclin D1 amplification/overexpression and p53 mutations were defined by increased nuclear staining, a method that has been validated in breast carcinoma by others (Zukerburg et. al. 1995; Alle et. al. 1998; Deng et. al. 1994). Loss of retinoblastoma (Rb) function was defined by a decrease in nuclear staining (Pietilainen et. al. 1995; Varley et. al. 1989; Geradts et. al. 1994); whereas, Her2/neu positivity was judged based on plasma membrane immunoreactivity (Paik et. al. 1991). An abnormal result was defined as follows: Cyclin D1 nuclear staining in $\geq$ 10% of cells; p53 nuclear staining in $\geq$ 5% of cells; Rb nuclear

staining in < 80% of cells; Her2/neu positive plasma membrane staining in any epithelial cell, graded on an intensity scale of 0-4. Note that the criterion for cyclin D1 is slightly more stringent than has been previously published (Van Diest et. al. 1997).

Vascular Score: Vessels were defined by immunohistochemistry for von Willebrand factor. Vascularity was examined by two methods. In all tissues the vascular score was determined for each histologic type of epithelium, as previously described (Heffelfinger et. al. 1996). Briefly, this is a quartile score representing the proportion of basement membrane for each alveolar or ductal unit that is surrounded by vessels. 1=less than 1/3, 2=1/3-2/3, and 3=greater than 2/3 of the circumference being surrounded by vessels. 4=complete encircling. Within a tissue a mean circumferential score (MCD) is calculated from the ductal or alveolar units within each histologic category. As others have noted, some types of pathologies induce neovascularization in the nearby stroma. This is particularly obvious in CIS. Therefore, in addition to the MCD, we determined a subjective mean vascular density (MVD) on a 0-4 scale based on observation of von Willebrand factor-stained vessels in the adjacent stroma, using a 0-4+ grading system, as defined by others (Guidi et. al. 1994).

Statistics: Data within each histologic subtype from each test were compared by Spearman Rank Order Correlation (SigmaStat, SPSS). Data for cyclin D1, Rb, and p53 were compared with vascularity, patient age at diagnosis, and the presence of concurrent or subsequent invasive disease (mean follow-up of 4.7 years). Mean vascular scores among groups were compared by one-way ANOVA and Tukey test, alpha = 0.05 (SigmaStat, SPSS, Chicago, IL). Proportions of proteins positive in each category were compared by z-test SigmaStat, SPSS, Chicago, IL).

Results:

Figure 1 shows examples of each immunologic stain from samples that were considered "positive" in these studies. Table I indicates the number of specimens that were positive (percent positive cases) for alterations in cyclin D1, Rb, p53, or Her2/neu for each histologic subtype. Table II shows the MCD (mean and standard deviation) for all histologic types in these same cases, as well as the MVD for cases of CIS.

Normal epithelium: Normal epithelium has an extremely low MCD because only rarely are vessels found along the basement membrane. Note that in no instance was normal epithelium found to be positive for cyclin D1, Rb or p53. However, there was one case in which there was low expression of p53 (1% of cells) and three cases in which there was low expression of cyclin D1 (2, 3, and 4% of cells). Rb was found in 100% of nuclei in all normal epithelium. Her2/neu immunoreactivity was generally cytoplasmic and therefore not considered "positive" by our criteria.

Proliferative epithelium: When all proliferative epithelium is grouped together, 15% were positive for Cyclin D1; 4%, for Rb; 2%, for p53; and 10%, for Her2/neu. In Table I these data are segregated into usual and atypical hyperplasia because there is a clear difference in the number of cases positive for cyclin D1

between these two groups. Proportionally more cases were positive for cyclin D1 in the group of atypias, 43% vs. 11%. On the other hand the rare cases of Rb underexpression and p53 overexpression are confined to the usual hyperplasias. Because AH is such a rare diagnosis, the number of positive cases is too small to formulate any conclusions.

We were surprised to find (albeit weak) plasma membrane staining for Her2/neu among the hyperplasias. Since commercial antibodies for Her2/neu are known for their variability in immunoreactivity (Bobrow et. al. 1996; Maia 1999), we re-tested these cases using a second antibody, CB11. In all cases the stain intensity was the same, but the staining was more diffusely cytoplasmic, with less immunoreactivity on the plasma membrane than in the original studies. In Figure 1 we show an example of the weak plasma membrane reactivity for Her2/neu in a hyperplastic epithelium from our original studies.

In situ carcinoma: 51% of all carcinomas in situ showed alterations in cyclin D1, whereas only 8 and 10% of cases showed alterations in Rb or p53, respectively. 30% of CIS showed immunoreactivity to Her2/neu. Consistent with its aggressive nature, comedo carcinoma had the preponderance of protein changes for all four genes. Cyclin D1 was the only protein found to have changes in comedo, non-comedo, and lobular carcinoma in situ. Changes in Rb and p53 were not found in

LCIS and were rarely found in non-comedo DCIS at 6% for both; whereas, Her2/neu was found in 20% of non-comedo DCIS, 100% of comedo CIS and never in LCIS. Therefore, among comedo CIS one or more alterations in protein expression was identified in 100% of cases and 50% of cases had alterations in expression of more than one protein. Among all cases of CIS, 19% had changes in expression of two or more of the proteins tested. Twenty-seven percent had no abnormal protein expression.

Vascularity and protein expression: There is a positive correlation between vascularity and cyclin D1 expression across all histologic subtypes indicating that both increase with disease progression. The number of positive samples within UH and AH were too few to make correlative statements regarding vascularity. However, at the stage of CIS several interesting correlations were identified. Rb positivity clearly bore no relation to MCD. However, there was a consistent trend for Rb positivity to be found exclusively in the CIS cases with the highest MVD. For instance, there is no Rb positivity in those cases with an MVD of 0, whereas, Rb is positive in 13.6% of cases in which vascularity is increased in the peripheral tissues (MVD>0). These numbers are too small for statistical evaluation. P53 positivity, on the other hand, correlates directly with both MVD and MCD. As shown in Figure 2a, p53 positive cases are more highly associated with an MCD above the mean than are cases which are p53 negative ($p=0.028$). The reverse is

also true in that cases with vascular scores above the mean were more likely to be p53 positive (22%) than p53 negative (3%) (Figure 2b). Figure 3 shows that these relationships are also true for p53 and MVD. Cyclin D1 expression is unrelated to MCD, but inversely related to MVD. For instance, among MVD negative cases, cyclin D1 is positive 90% of the time; data for an MVD of 1-2 are positive 48%, and for an MVD of 3-4, 36% of cases, significant at p=0.038. Figure 4 shows the data broken down by cyclin D1 positive vs negative cases. Therefore, while p53 has a positive correlation to both MCD and MVD, cyclin D1 is inversely correlated to only MVD. On the other hand, Rb may be positively correlated with MVD. Her2/neu has no correlation with MCD or MVD.

As expected, comedo CIS had the greatest MCD and MVD. In addition, comedo CIS had the greatest frequency of aberrant expression of cyclin D, Rb, P53, and Her2/neu. Therefore, we asked whether the correlation between protein expression and vascularity are simply due to the fact that each are more common among comedo CIS. For p53 there were too few positive cases among patients with CIS to make conclusions. Among comedo cases, 60% of p53 negative cases had an MCD greater than the mean; whereas, 67% of p53 positive cases had an MCD greater than the mean, clearly not a large difference. However, only 40% of p53 negative cases had an MVD greater than the mean, whereas 100% of p53 positive cases had an MVD greater than the mean, a much more convincing

correlation. Therefore, at least for MVD, p53 positivity still correlates directly within the subset of comedo CIS. With cyclin D1 the story is similar. Whether in the non-comedo or comedo group, cyclin D shows no consistent correlation with MCD. For non-comedo CIS cyclin D1 negative vs. positive cases, 39 vs 29% have an MVD above the mean. For comedo CIS cyclin D1 negative vs. positive cases, 100 vs. 40% have an MVD above the mean. Again the trends are reflective of the larger data set. However, for all these subset analyses the numbers are too small to have statistical power.

Level of protein expression and disease progression: Table III shows the mean and range in level of expression as percent positive cells (for cyclin D1, Rb, and p53) and stain intensity (for Her2/neu) among the cases that were positive for each protein. Note that for cyclin D1 the number of positive cases increases with progression (Table I) but the level of expression within individual cases (as defined by percent positive cells) does not increase (Table III). For instance, the mean percent of positive cells for cyclin D1 was 38 within both the proliferative lesions and comedo carcinoma. A similar situation is true for Rb and p53. Note also from the ranges that in no case was Rb 0%, or cyclin D1 or p53, 100%.

Because of the possibility that alterations in the expression of one of these proteins may be related to the presence of concurrent disease (as in mastectomy

specimens) or pre vs. post-menopausal disease progression, we tested these data for correlation to age at diagnosis and presence of concurrent or subsequent invasive disease. No correlation was identified.

Discussion:

We examined pre-invasive breast pathologies in archival tissues from 89 patients for the expression of cyclin D1, p53, Rb and Her2/neu protein to determine when in the progressive pathway to invasion each alteration in these proteins occurs and to correlate these changes with the level of vascularity. We found that cyclin D1 expression, as defined by the number of cases with increased cyclin D1 protein immunoreactivity, increases with disease progression, being absent in normal epithelium, and present in a greater percentage of cases up to carcinoma in situ, in which it is found in over half of the cases. These data show a strong positive correlation with MCD, which also increases with disease progression. Interestingly, the range and mean of cyclin D1 positivity within individual cases in each histologic group (percent positive cells) do not appear to change with progression. We interpret these data to mean that vascularity in the earliest pre-invasive pathologies increases with progression independent of cyclin D1 status. This is consistent with our analysis of cyclin D1 expression in CIS in which there is an inverse correlation with MVD.

Similar to Alle et al, we found that cyclin D1 expression is increased in usual hyperplasia (Alle et. al. 1998). Although it is somewhat more common within the class of atypical hyperplasia, nothing about the level of its expression would be useful for discriminating usual from atypical hyperplasia. Furthermore, we could

not discriminate between hyperplasia and carcinoma in situ based on these data, as has been suggested by others (Weinstat-Saslow et. al. 1995; Gillett et. al. 1998), since a significant proportion of both groups had similar levels of overexpression. Overall the level of positive cyclin D1 staining in in situ carcinoma is similar to that found in other studies (Alle et. al. 1998; Gillett et. al. 1998). Whether these increased protein levels are due to gene amplification, we have not yet determined. It has been demonstrated previously that not all cases of positive immunoreactivity are due to gene amplification (Michalides et. al. 1996; Zhang et. al.1994).

Criteria for positive Rb and p53 immunoreactivity in this study are similar to those reported by others (Pietilainen et. al. 1995; Barbareschi 1996). For p53 in particular, there has been extensive correlative work between immunologic and genetic analyses which indicate that positive immunoreactivity may not always denote a gene mutation (Thompson et. al. 1992; Visscher et. al. 1996). However, the higher the percent of cells staining, the more likely a mutation will be identified (Doglioni et. al. 1995). In these cases the mechanism of increased p53 immunoreactivity and Rb loss are not known. Alterations in Rb and p53 protein expression were rarely found in epithelial hyperplasia and non-comedo DCIS, but the majority of abnormalities occurred within the comedo carcinomas. These data are consistent with the levels of p53 mutation reported by others (Walker et. al.

1991; Elledge et. al. 1994). To our knowledge this is the first report of decreased Rb expression in pre-invasive lesions. Among our cases of LCIS, none showed changes in Rb or p53. However, the fact that nearly three quarters of the carcinomas in situ contained one or more changes in these four proteins is consistent with the observation that they are genetically advanced lesions (Visscerh et. al. 1993; Zuang et. al. 1995).

Several aspects of our Her2/neu data are consistent with data reported by others (Gusterson et. al. 1988; Van de Vijver 1988; Iglehart et. al. 1990; Liu et. al. 1992; Allred et. al. 1992; Porter et. al. 1991). The overall level of Her2/neu expression in CIS was 30%, with 100% positivity in cases of comedo CIS and no expression in cases of LCIS. As expected, nearly all cases of CIS and invasion had robust (3-4+) plasma membrane immunoreactivity. However, we also identified focal plasma membrane Her2/neu immunoreactivity in UH and AH. Restaining these with a second antibody confirmed that some plasma membrane staining was present.

Among CIS cases, increased cyclin D1 positivity is inversely associated with MVD. We interpret these data to mean that changes in cyclin D1 positivity are one of many genetic changes that occur during progression, but the level of expression is not driving the phenotypic features that we associate

morphologically as vascularity. Indeed the genetic pathway leading to increased cyclin D1 expression may be less angiogenic at the stage of CIS than some other genetic pathways. For example, p53 shows a clear positive correlation with both MVD and MCD. Although there are known mechanisms through which p53 mutations regulate angiogenic potential (Rastinejad et. al. 1989), it is not known whether these mechanisms operate in pre-invasive breast pathologies. Our attempts to examine these tissues for thrombospondin 1, an angiogenic inhibitor transcriptionally regulated by p53 (Dameron et. al. 1994), showed no reactivity with normal breast epithelium (data not shown).

Although correlative, these data suggest that as early as CIS, genetic changes may regulate specific types of tissue vascularity. It was not expected that MCD and MVD would have unique associations with specific oncogenes and tumor suppressor genes. Engels et al have suggested that the two types of vascularity seen in CIS may be differentially regulated (Engels et. al. 1997). These data support this hypothesis. Having identified these associations, we must now identify mechanisms through which p53 and cyclin D1 may be altering the vascularity in these tissues.

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Table I. Percent Positive Cases in Each Histologic Subtype

	Cyclin D1	Rb	P53	Neu
Normal	0	0	0	-
UH	11	4	2	3
AH	43	0	0	8
Non-com	49	6	6	20
Comedo	57	29	43	100
Lobular	67	0	0	0

Percent positive cells for each histologic category were determined from the proportion of epithelial nuclei that showed specific staining. Histologic categories include normal epithelium (Normal), UH, AH, non-comedo ductal CIS (Non-com), comedo ductal CIS (Comedo), and lobular CIS (Lobular). Cyclin D1 and p53 were considered positive if 10 or 5 percent of nuclei or greater were stained, respectively. Rb was considered positive if less than 80% of nuclei were stained. Neu was considered positive if any cluster of cells showed plasma membrane immunoreactivity of $\geq$ 1+.

Table II. Vascularity by Histologic Subtype

	MCD	MVD
Normal	0.18 ± 0.15	
UH	1.01 ± 0.42	
AH	0.92 ± 0.39	
Non-com	1.66 ± 0.83	1.36 ± 1.15
Comedo	2.32 ± 0.77*	2.63 ± 1.19*
Lobular	1.67 ± 0.46	

Shown here is the mean ± standard deviation of MCD for each histologic subtype. MVD is show for ductal CIS. Histologic categories include normal epithelium (Normal), UH, AH, non-comedo ductal CIS (Non-com), comedo ductal CIS (Comedo), and lobular CIS (Lobular). Note that the MCD in all categories is statistically greater than in normal epithelium ($P<0.001$). The * denotes that the MCD of comedo is significantly greater than non-comedo CIS ($p=0.41$); similarly, the * denotes the same relationship for MVD ($p=0.006$).

Table III. Level of Expression (Percent Positive Cells or Stain Intensity) Among Positive Cases in Each Histologic Subtype

	Cyclin D1	Rb	P53	Her2/neu
Normal	-	-	-	-
UH	38 (15-44)	60(60)	80 (80)	1.50 (1-2)
AH	25 (20-30)	0	-	2.00 (2)
Non-com	57 (10-75)	57(50-65)	48 (10-80)	2.08 (1-4)
Comedo	38 (12-85)	75(75)	77 (50-95)	3.6 (2-4)
Lobular	20 (10-30)	0	-	-

Reported here are the mean and range of percent positive cells (Cyclin D1, Rb, and p53) or stain intensity (Her2/neu) within individual cases for each antibody, segregated by histologic subtype. Histologic categories include normal epithelium (Normal), UH, AH, non-comedo ductal CIS (Non-com), comedo ductal CIS (Comedo), and lobular CIS (Lobular).

Figure 1. Positive Immunologic Stains. Shown are examples of positive immunohistochemical stains for cyclin D1 (a), retinoblastoma (b), p53 (c), and Her2/neu (d). Panels a-c are CIS. Panel d is hyperplasia. Arrowheads in panels a and c highlight the positive nuclear immunoreactivity. The arrows in panel b indicate cells that lack immunoreactivity to Rb. The plasma membrane immunoreactivity for Her2/neu is highlighted by arrows in panel d.

Relation of p53 and MCD.

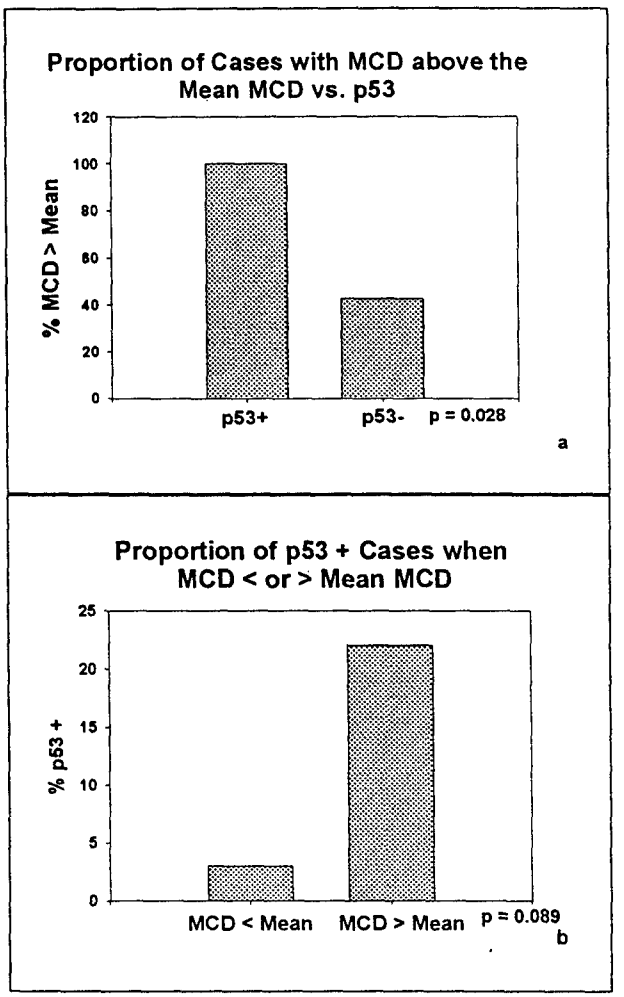

Figure 2. (a) Shown are the percent of p53 positive CIS cases in which the MCD is greater than the mean MCD for all CIS cases. These proportions are statistically different (p=0.028). (b) Shown is the inverse relation, that is the percent of p53 positive CIS cases that have an MCD below or above the mean MCD for all CIS cases.

Relation of p53 and MVD.

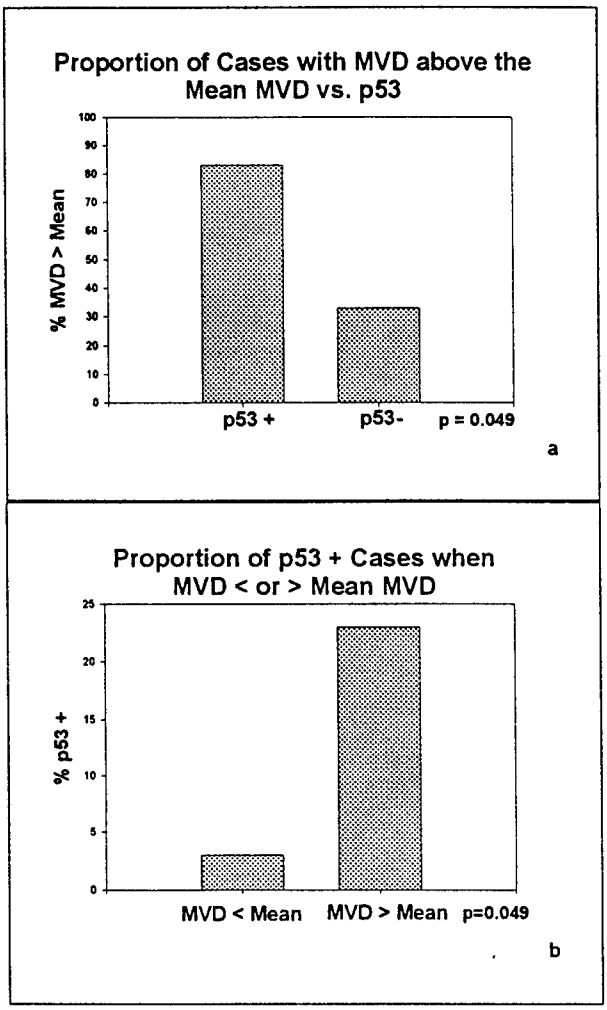

Figure 3. (a) Shown are the percent of p53 positive CIS cases in which the MVD is greater than the mean MVD for all CIS cases. These proportions are statistically different (p=0.049). (b) Shown is the inverse relation, that is the percent of p53 positive CIS cases that have an MVD below or above the mean MVD for all CIS cases. Again these proportions are different (p=0.049).

Relation of Cyclin D1 and MVD

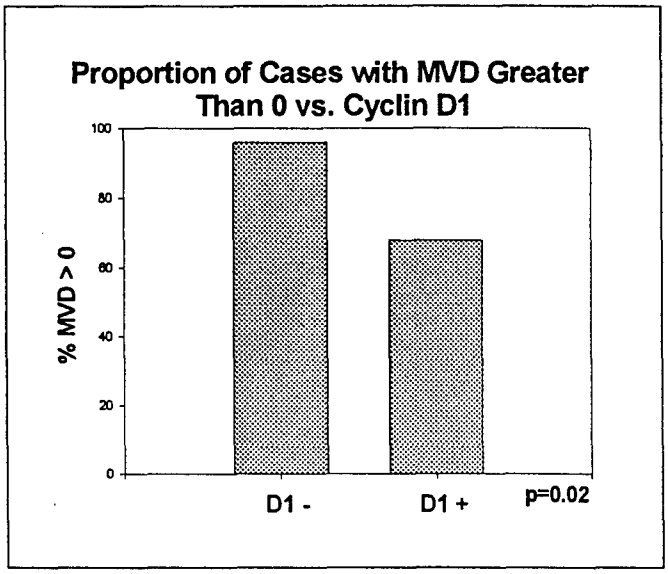

Figure 4. Shown are the percent of either cyclin D1 positive or negative CIS cases in which the MVD was greater than the mean MVD for all CIS cases. These proportions are significantly different (p=0.02).

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Reports to be changed to "Approved for public release; distribution unlimited"

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